

Rec'd PCT/PTO 10 APR 2005

PCT/NL

03 / 00703



Europäisches
Patentamt

European
Patent Office

Office européen
des brevets

REC'D 05 NOV 2003

WIPO PCT

101531855

Bescheinigung

Certificate

Attestation

Die angehefteten Unterlagen stimmen mit der ursprünglich eingereichten Fassung der auf dem nächsten Blatt bezeichneten europäischen Patentanmeldung überein.

The attached documents are exact copies of the European patent application described on the following page, as originally filed.

Les documents fixés à cette attestation sont conformes à la version initialement déposée de la demande de brevet européen spécifiée à la page suivante.

Patentanmeldung Nr. Patent application No. Demande de brevet n°

02079328.7

BEST AVAILABLE COPY

**PRIORITY
DOCUMENT**

SUBMITTED OR TRANSMITTED IN
COMPLIANCE WITH RULE 17.1(a) OR (b)

Der Präsident des Europäischen Patentamts;
Im Auftrag

For the President of the European Patent Office

Le Président de l'Office européen des brevets
p.o.

R C van Dijk



Anmeldung Nr:
Application no.: 02079328.7
Demande no:

Anmeldetag:
Date of filing: 17.10.02
Date de dépôt:

Anmelder/Applicant(s)/Demandeur(s):

Pharming Intellectual Property BV
Archimedesweg 4
2333 CN Leiden
PAYS-BAS

Bezeichnung der Erfindung/Title of the invention/Titre de l'invention:
(Falls die Bezeichnung der Erfindung nicht angegeben ist, siehe Beschreibung.
If no title is shown please refer to the description.
Si aucun titre n'est indiqué se referer à la description.)

Protein modification

In Anspruch genommene Priorität(en) / Priority(ies) claimed /Priorité(s)
revendiquée(s)
Staat/Tag/Aktenzeichen/State/Date/File no./Pays/Date/Numéro de dépôt:

Internationale Patentklassifikation/International Patent Classification/
Classification internationale des brevets:

C12P/

Am Anmeldetag benannte Vertragstaaten/Contracting states designated at date of
filing/Etats contractants désignées lors du dépôt:

AT BE BG CH CY CZ DE DK EE ES FI FR GB GR IE IT LI LU MC NL PT SE SK TR

17.10.2022

(102)

PROTEIN MODIFICATION

Field of the invention

5

The present invention relates to the modification of glycosylated compounds, more specifically to the modification of recombinantly produced glycosylated compounds to increase their circulatory lifetime in the blood.

10 Background of the invention

15

Glycoproteins are a conjugated form of proteins containing one or more covalently bound carbohydrates. Protein-linked carbohydrates may be classified into two groups depending on the nature of the linkage between the glycan and the protein, viz. N-linked carbohydrates which are attached to the free amino group of asparagine residues and O-linked carbohydrates which are linked to the hydroxyl group of threonine and serine residues.

20

It is well known that the half-life of glycoproteins, *i.e.* the circulatory lifetime in the blood, is highly dependent on the composition and structure of its N-linked carbohydrates. For instance, the removal of sialic acid groups from the carbohydrates of glycoproteins will result in rapid clearance of these glycoproteins from circulation (Morell et al. (1971) J. Biol. Chem. 246. 1461), since the desialylated glycoproteins are recognised by various carbohydrate receptors in the body. Examples of such carbohydrate receptors involved in clearance are the asialoglycoprotein receptor and the mannose receptor on liver cells. The same phenomenon is observed in the case of recombinantly produced human proteins, such as human proteins produced in Chinese hamster ovary (CHO) cells or in transgenic animals, which, in general, contain less sialic acids groups than their non-transgenic counterparts.

25

As to date, it is generally accepted that N-linked carbohydrates dominate the pharmacokinetic properties of a glycoprotein. Therefore, the preferred strategy to improve the half-life of a glycoprotein has been modification of its N-linked carbohydrate groups, through sialylation or removal of terminal galactose residues.

DETAILED DESCRIPTION

- The present invention relates to a method for changing the half-life of a glycosylated compound by the modification of an O-linked carbohydrate. In this context, half-life is
- 5 defined as the circulatory lifetime in the blood. In this context "carbohydrate" refers to both monosaccharides and oligosaccharides. As to date, it was thought that half-life was highly dependent on and mainly dominated by the composition and structure of N-linked carbohydrates. The inventors demonstrate that also O-linked carbohydrates may govern the half-lives of glycosylated compounds. This is of direct relevance to
- 10 therapeutic application of glycosylated compounds that are administered parenterally, because according to the method of the present invention half-lives may be dramatically prolonged.
- The method of the invention may be used to either reduce or increase the half-life of a
- 15 glycosylated compound. In a preferred embodiment of the invention, the modification at the O-linked carbohydrate is used to extend the half-life of a glycosylated compound. By this improvement, the half-life of the modified glycosylated compound is increased by at least 10%, preferably by at least 30%, 50% or 70% as compared to the unmodified compound. Most preferred is that the value of the half-life of the
- 20 unmodified glycosylated compound has increased to at least twice, three times or four times the original value of the half-life.
- The modification of the O-linked carbohydrate is preferably carried out enzymatically by using an enzyme preparation. The enzyme preparation may comprise one enzyme or
- 25 a mixture of enzymes. These enzymes may have a varying degree of purity. They may be purified or substantially pure, but this is not an absolute requirement.
- Both *in vivo* and *in vitro* modification protocols may be used to modify the O-linked carbohydrates. Examples of *in vivo* modification include, but are not limited to,
- 30 modifications that take place in cell culture systems or in transgenic animals.

The modification of the O-linked carbohydrates is concentrated on the capping or removal of terminal galactose residues, interfering with the binding to receptors

involved in clearance and therefore leading to a prolonged circulatory life time in the blood. Suitable enzymes include, but are not limited to, sialyltransferases for capping terminal galactose. Examples of enzymes which are useful for the removal of terminal galactose are galactosidases and endo-acetylgalactosaminidases (O-glycosidase).

- 5 Galactosidases are capable of removing terminal galactose from either N- or O-linked carbohydrates, whereas endo-acetylgalactosaminidases hydrolyse the covalent linkage between the polypeptide and galactosamine (O-linked to either serines or threonines) of non-sialylated $\text{Gal}\beta 1,3\text{GalNAc}$ structures. In both cases the number of exposed galactose residues will be reduced and will therefore enhance the circulatory life time
10 of the glycoprotein.

A preferred way of modification of the O-linked carbohydrate group is sialylation. In general, sialylation involves the transfer of sialic acid from a sialic acid donor to a carbohydrate group on a glycosylated compound by the action of a sialyltransferase.

- 15 This may either take place *in vivo* (by co-expression of the glycosyltransferase in the glycoprotein expression system) or *in vitro*. The sialyltransferase may be recombinantly produced or isolated from a sialyltransferase source. A preferred example of a sialyltransferase to be used in the method of the invention is ST3Gal I, preferably in combination with ST3Gal III. ST3Gal I specifically transfers a sialic acid to the
20 terminal galactose of $\text{Gal}\beta 1,3\text{GalNAc}$ epitopes which is the core structure of mucin type O-linked carbohydrates, whereas ST3Gal III is specific for lactosamine units ($\text{Gal}\beta 1,4\text{GlcNAc}$) often occurring in complex and hybrid type N-linked carbohydrates. The method described herein may be used to improve the pharmacokinetic properties of any glycosylated compound bearing mucin type O-linked carbohydrates.

- 25 Alternatively, the circulatory half-life of a glycosylated compound may be extended through modification of its O-linked carbohydrate groups by removing part or all of these carbohydrates. Preferably only part or all of the non-sialylated O-linked carbohydrates are removed.

- 30 Any glycosylated compound of which the half-life has to be modified may be used in the method according to the invention. Preferably, the method is used for recombinantly produced glycoproteins. The method is extremely useful for improving

the half-life of a recombinantly produced glycoprotein that is intended to be administered parenterally.

- In this context "recombinantly produced glycoproteins" refers to glycoproteins which
- 5 are produced by cells which replicate a heterologous nucleic acid, or expresses a peptide or protein encoded by a heterologous nucleic acid. The heterologous nucleic acid typically contains genes which are not found in the native or natural form of the cell or which may be found in such cell but which have been modified or manipulated.
- 10 In the context of this application, recombinantly produced glycoproteins are preferably produced in mammalian cell culture systems or in transgenic animals, such as in goat, sheep and cattle. The glycoprotein may be obtained from these production systems in a manner known per se for isolating and/or purifying recombinantly produced proteins. *In vitro* modification may take place during or after isolation or purification. If it is
- 15 implemented during purification, it has the advantage that modification additives may be removed during downstream processing.

- In one embodiment, recombinant human C1 inhibitor (rhC1INH), purified from the milk of transgenic rabbits, is sialylated *in vitro* by using a mixture of recombinantly produced sialyltransferases.

EXAMPLES

Experimental

- 25
- Sialic acid determination**
- Sialic acids on rhC1INH samples produced in rabbits were quantified in the following way: sialidase from *Arthrobacter ureafaciens* was added to rhC1INH and samples were incubated for 1 h at 37°C. The amount of released sialic acid was quantitated
- 30 determined on HPAEC-PAD after adding 3-deoxy-D-glycero-D-galacto-2-nonulosonic acid (KDN, Toronto research Chemicals) as an internal control.

SDS-PAGE and inhibitory activity

Non-reduced and reduced SDS-PAGE was performed using the Novex system as recommended by the manufacturer. Proteins were visualized by silver staining. Inhibitory activity of rhC1INH, either before or after *in vitro* sialylation, was determined according to a standard procedure with the target protease C1s in the presence of a synthetic chromogenic substrate. After determining the rhC1INH antigen concentration by an ELISA assay, the specific activity in mU/mg protein was calculated.

10 N-linked glycosylation profiling

N-linked glycosylation profiling was performed according to an in-house method. Briefly, rhC1INH was diluted in 25 mM sodium phosphate, 62 mM EDTA, pH 7.2 containing 5 mg/ml N-octylglucoside and boiled for 2 min. Subsequently, N-glycosidase F was added and samples were incubated for 45 h at 37°C. Samples were rotated for 5 min at 14,000 rpm and supernatant was analyzed on a CarboPac PA-1 column with CarboPac PA-100 guard, pre-equilibrated in 150 mM NaOH. Carbohydrates were eluted with a 0-175 mM sodium acetate gradient in 150 mM NaOH at 1 ml/min in 30 min.

20 O-linked glycosylation profiling

The O-linked carbohydrates were removed from rhC1INH by β -elimination after the N-linked carbohydrates had been removed from the rhC1INH preparations. Therefore, 200 μ g of rhC1INH was treated with N-glycosidase F as described above, with the exception that samples were digested for 17 h instead of 48 h. After deglycosylation, samples were mixed with three volumes of 96% (v/v) ethanol and incubated for 10 min on ice before rotation for 5 min at 15000 rpm at 4°C. Protein pellets were twice dissolved in water and precipitated again with ethanol. After the second wash, pellets were dried in a SpeedVac at room temperature and subsequently dissolved in 100 μ l 1.0 M NaBH₄, 50 mM NaOH and incubated for 17 h at 45°C. β -elimination was stopped by the addition of HAc (0.8 M final concentration) on ice. Samples were dried in a SpeedVac and washed three times with 1% HAc in methanol. After the third wash, pellets were dissolved in 100 μ l water and samples were loaded on a Biorad

- AG50WX12 column (1 ml packed beads per sample) pre-equilibrated in water. Columns were eluted with three column volumes of water. Fractions were dried in the SpeedVac and resuspended in 100 µl of water. Samples were loaded on a CarboPac PA-1 column with CarboPac PA-100 guard, preequilibrated in 150 mM NaOH.
- 5 Carbohydrates were eluted with a 0-250 mM sodium acetate gradient in 150 mM NaOH at 1 ml/min in 30 min.

Example 1 *In vitro* sialylation protocol A

- 10 *In vitro* sialylation was carried out by Neose Technologies, Inc (La Jolla, CA) on batch 04i00011 recombinant human C1 inhibitor produced in the milk of transgenic rabbits, in 50 mM Tris, 0.15 M NaCl, 0.05% NaN₃, pH 7.2 by the addition of the sialyltransferase ST3Gal III in combination with CMP-Sialic acid. After incubation for 16 h at 32°C samples were frozen in liquid nitrogen and shipped. Samples were thawed
- 15 and dialysed in Spectrapor membranes with a cut-off of Mr 25,000 against 10 mM sodium phosphate, 0.15 M NaCl, pH 7.4 (PBS) before applying the assays described in this application.

Determination of the amount of sialic acids on rhC1INH revealed that it had increased

20 from 7 mol sialic acid/mol rhC1INH to 9 mol/mol. *In vitro* sialylation had no impact on the protease inhibitory activity of the protein and did not cause any degradation or aggregation.

N-linked glycosylation profiling of rhC1INH showed that *in vitro* sialylation caused a

25 significant increase, *i.e.* about 7-fold, in the amount of double-sialylated structures. Not all the mono-sialylated structures could be converted into double sialylated structures, suggesting that the remaining structures did not contain acceptor sites for the sialyltransferase(s).

30 O-linked glycosylation profiling of rhC1INH showed only a minor increase in the amount of sialylated Gal-GalNAc, indicating that only a minor portion of the sialic acids had been incorporated into the O-linked carbohydrates.

Example 2 *In vitro* sialylation protocol B

5 *In vitro* sialylation on recombinant human C₁-Inhibitor batch 04i00011 in 50 mM Tris, 0.15 M NaCl, 0.05% NaN₃, pH 7.2 was performed as described in Example 1, but now a mixture of sialyltransferases ST3Gal III and ST3Gal I was used.

Determination of the amount of sialic acids on rhC1INH revealed that it had increased from 7 mol sialic acid per mol rhC1INH to 28 mol/mol.

10 N-linked glycosylation profiling of rhC1INH showed that *in vitro* sialylation caused a significant increase, i. e about 7-fold, in the amount of double-sialylated structures. Also in this case, not all mono-sialylated structures could be converted into double-sialylated structures.

15 The O-linked glycosylation profiling of rhC1INH showed that the majority of the sialic acids had been incorporated into the O-linked carbohydrates, i.e. the amount of mono-sialylated Gal β 1,3GalNAc increased approximately 10-fold.

20 Results are summarised in Table 1 (N-linked glycosylation profiling) and Table 2 (O-linked glycosylation profiling) and clearly show that there is no difference in the N-linked profile of both samples, whereas the O-linked profile showed significant differences. Hence, the most important difference between sample rhC1INH-A and rhC1INH-B is in the degree of sialylation of the O-linked carbohydrates.

Table 1
Relative peak areas of the N-linked glycan HPAEC-PAD profile of *in vitro* sialylated recombinant human rhC1INH.

Charge group	Relative peak area (%)		
	rhC1INH 04i00011	rhC1INH- protocol A	rhC1INH- protocol B
Uncharged	20	17	18
Mono-charged	74	49	45
Double-charged	5	34	37

5

10 **Table 2**
Relative peak areas of the O-linked glycan HPAEC-PAD profile of *in vitro* sialylated rhC1INH.

Peak	Relative peak area (%)			
	rhC1INH	rhC1INH-A	rhC1INH-B	Plasma C1 Inhibitor
Uncharged	85	77	34	11
Monocharged	10	19	59	78
Double charged	4	4	7	11

Example 3 Pharmacokinetics of modified rhC1INH

Rats were anaesthetised by subcutaneous injection of hypnorm/midazolam and the abdomen was opened. The test items, i.e. rhC1INH samples, were injected via the tail vein or the vena cava or the vena penis. At the indicated times, blood samples of approximately 0.2 ml were taken from the inferior vena cava and transferred to eppendorf vials with 10 µl of 0.5 M EDTA in PBS. The samples were centrifuged for 5 min at 3500 × g and 100 µl plasma of each sample was stored at -20°C upon analysis. The plasma samples were analysed by using an ELISA for the detection of rhC1INH. Recombinant human C1INH had a plasma circulatory half-life of 16 ± 3.7 min, whereas rhC1INH-A and rhC1INH-B had a half-life of 25 ± 3 and 75 ± 14 min, respectively. The half-life of rhC1INH-B was similar to what we measured previously for human C1 Inhibitor isolated from human plasma, i.e 75 ± 14 min.

These results clearly show that improvement of half-life was obtained through modification of the O-linked carbohydrates. This improvement may be obtained over the unmodified protein (almost 5 times the original value), but also over the (N-linked) modified protein (3 times the N-linked value). This degree of improvement is a new observation, which has never been reported for O-linked carbohydrates before.

Table 5
Half-lives of different C1 INH samples

20

	Half-life (min)
rhC1INH	16 ± 3.7
rhC1INH ST3Gal III	25 ± 3
rhC1INH ST3Gal III + ST3Gal I	75 ± 14
plasma C1 Inhibitor	75 ± 14

Example 4 *In vitro* modification of rhC1INH O-linked carbohydrates by using Endo- α -N-Acetylgalactosaminidase

5 Removal of non-sialylated O-glycans from rhC1INH was accomplished by using a commercially available recombinant Endo- α -N-Acetylgalactosaminidase (O-glycosidase, Prozyme, which is specific for the hydrolysis of Gal β 1,3GalNAc α -Ser/Thr- structures). To this end, different amounts of O-glycosidase, ranging from 0.125-3.25 mU, were added to 200 μ g of rhC1INH in 40 μ l of a 20 mM phosphate buffer of pH 5.0. The mixture was incubated overnight at 37 °C after which the protein was precipitated and washed three times with 70 % ethanol to remove the released Gal β 1,3GalNAc. Subsequently, the samples were subjected to O-glycan profiling by using HPAEC-PAD as described in the experimental section. The chromatograms of unmodified and modified rhC1INH showed that O-glycosidase treatment significantly 15 reduced the amount of Gal β 1,3GalNAc on rhC1INH. The Gal β 1,3GalNAc peak was reduced to approximately 25% as compared to the unmodified rhC1INH. Moreover, O-glycosidase treatment did not affect the protease inhibitory activity of rhC1INH nor did it cause aggregation or degradation. The reduction in the number of non-sialylated O-glycans in rhC1INH is expected to lead to improved pharmacokinetics of the 20 modified product.

The method described herein may be used to improve the pharmacokinetic properties of any glycosylated compound bearing mucin type O-linked glycans.

17.10.2002

CLAIMS

(102)

1. Method for changing the half-life of a glycosylated compound, which method comprises the modification of an O-linked carbohydrate.
5
2. Method according to claim 1 wherein the modification is a sialylation of an O-linked carbohydrate.
- 10 3. Method according to claim 1 wherein the modification comprises the removal of one or more non-sialylated O-linked carbohydrates.
4. Method according to claim 3, wherein the non-sialylated O-linked carbohydrate is galactose.
- 15 5. Method according to claim 1-4 wherein the modification is an *in vitro* modification.
6. Method according to claim 1-5 wherein the O-linked carbohydrate is modified by *in vitro* incubation with an enzyme preparation which comprises one or more enzymes.
- 20 7. Method according to claim 1-6 wherein the enzyme preparation comprises recombinant ST3Gal III and ST3Gal I.
8. Method according to claim 1-7 wherein the plasma circulatory half-life is improved, preferably has at least doubled in comparison to the unmodified compound.
25
9. Method according to claim 1-8 wherein the glycosylated compound is a glycoprotein, preferably a C1 Inhibitor, more preferably human C1 Inhibitor.
- 30 10. Method according to claim 1-9 wherein the C1 Inhibitor is produced by recombinant DNA technology.

A B S T R A C T

- 5 The present invention relates to a method for changing the half-life of a glycosylated compound by the modification of its O-linked carbohydrates. This modification is preferably carried out enzymatically and aimed at extending the half-life of the compound. Both in vivo and in vitro modification protocols may be used.

10

EPO - DG

17. 10. 2002

(102)

PCT Application

NL0300703



This Page is inserted by IFW Indexing and Scanning
Operations and is not part of the Official Record

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- BLACK BORDERS
- IMAGE CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT OR DRAWING
- BLURED OR ILLEGIBLE TEXT OR DRAWING
- SKEWED/SLANTED IMAGES
- COLORED OR BLACK AND WHITE PHOTOGRAPHS
- GRAY SCALE DOCUMENTS
- LINES OR MARKS ON ORIGINAL DOCUMENT
- REPERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY
- OTHER: _____

**IMAGES ARE BEST AVAILABLE COPY.
As rescanning documents *will not* correct images
problems checked, please do not report the
problems to the IFW Image Problem Mailbox**